

**The Nuclear Receptor Corepressor Deacetylase Activating Domain is  
Essential for Repression by Thyroid Hormone Receptor**

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## **ABSTRACT**

Nuclear receptor corepressor (N-CoR) mediates repression by thyroid hormone receptor (TR) as well as other nuclear hormone receptors and transcription factors. N-CoR contains several domains (RDs) that repress transcription when fused to a heterologous DNA-binding domain, but their relative importance in the full length N-CoR molecule is unknown. Here we addressed this important issue by depleting N-CoR in human cells and replacing it with mutant and wild type murine N-CoR. Although the N-terminal RD has binds TBL1/TBLR1 and mSin3, deletion of this region did not affect the ability of N-CoR to mediate repression by TR. By contrast, deletion of the deacetylase activating domain (DAD) that binds and activates histone deacetylase 3 dramatically hampered N-CoR's function as a TR corepressor. Introduction of a single amino acid mutation in the DAD similarly disabled the corepressor function of N-CoR. Thus, the DAD domain of N-CoR is singularly essential for repression by TR.

## Introduction

Nuclear hormone receptors (NRs) regulate gene transcription in response to lipophilic ligands that include hormones, vitamin derivatives, and other metabolites (1). Many NRs are constitutively nuclear and bind to target genes in the absence as well as in the presence of ligand. Liganded NRs activate transcription by recruiting coactivator molecules such as the steroid receptor coactivator (SRC) family and thyroid receptor associated protein 220 (TRAP220)[for review, see (2)]. By contrast, unliganded NRs bind to corepressors that actively silence basal transcription (3, 4).

The biological relevance of the ligand-independent repression function has been particularly well-documented for thyroid hormone receptors (TR). In mice, genetic deletion of TR increases the basal expression of thyroid-responsive genes (5, 6). Moreover, the severity of resistance to thyroid hormone in humans bearing mutations in one TR $\beta$  allele best correlates with constitutive corepressor binding by the abnormal gene product (7, 8). NR corepressor binding by leukemogenic fusion proteins including APL-RAR $\alpha$ , PLZF-RAR $\alpha$ , and AML-ETO has also been implicated in the pathogenesis of myeloid leukemias (9-16).

The best characterized NR corepressors are Nuclear Receptor Corepressor (N-CoR)(17) and Silencing Mediator of Retinoid and Thyroid receptors (SMRT)(18). N-CoR and SMRT are large proteins (~270 kDa) that are highly related to one another and meet the important criteria of 1) binding to unliganded NRs with considerably higher affinity than to ligand bound NRs (17, 18), 2) possessing intrinsic transcriptional repressive activities (17, 18), 3) being required for repression by unliganded NRs in living cells (19, 20). Binding to unliganded NRs is achieved via the short amphipathic helical corepressor-NR (CoRNR) motifs contained within the C-terminal region of the corepressors (21-23). The CoRNR motifs are highly conserved across

mammalian species for both N-CoR and SMRT, more so than between the two corepressors. As such, some NRs prefer one corepressor over another (24-27). Notably, TR binds preferentially to N-CoR over SMRT (25, 26, 28-30). Furthermore, knockdown of N-CoR in human cells is sufficient to cripple the repression function of unliganded TR (19, 20, 26).

The ability of N-CoR to repress transcription is thought to be conferred by the N-terminus of the molecule, which contains several polypeptides that function as autonomous repression domains (RDs) when fused to heterologous DNA binding proteins (Fig. 1a)(17). One function of the RDs is to usher histone deacetylase (HDAC) activity to sites of N-CoR recruitment, thereby promoting a repressive chromatin environment. The most N-terminal, RD1, binds to histone HDACs 1 and 2 via the Sin3 corepressor (31-33). RD1 has also been implicated in the binding of core components of the N-CoR repression complex, namely Transducin Beta-Like Protein 1 (TBL1) (34) and its close relative TBLR1 (35), as well as G-Protein Suppressor 2 (GPS2)(36). RD2 has not yet been implicated in HDAC recruitment. RD3 binds to Class II HDACs 4, 5, and 7 (37, 38), although the enzymatic activity of these HDACs appears to rely on association with HDAC3 (39).

Between RD1 and RD2 is the region that is most highly conserved between N-CoR and SMRT, as well as the *Drosophila melanogaster* homologue, SMRTER (40). Although not originally identified as an RD, this region of the molecule also acts as a potent, autonomous repression domain (41). This function involves a pair of SANT motifs (SANT1 and SANT2) named after several transcriptional regulatory proteins in which they are found: Snf3, ADA2, N-CoR, and TFIIB (42). SANT1 in N-CoR is critical for interaction with HDAC3, a component of the core N-CoR repression complex (41). The enzymatic activity of HDAC3 actually requires binding of N-CoR (or SMRT)(36, 41); hence this region has been named the Deacetylase Activation Domain (DAD). N-CoR's SANT2 augments the repressive activity of SANT1/HDAC3 by acting

as a histone interaction domain (HID)(43); histone binding is also a property of SANT-containing domains in ADA2 and ISWI (44, 45).

Previous studies have shown that HDAC3 but not HDACs 1, 2, or 4 are recruited to target genes by TR, and that HDAC3 but not the other HDACs is required for repression by TR (20). However, understanding of the specific contributions of individual RDs as well as the DAD and HID has been limited by the abundance of N-CoR in most mammalian cells. Here we have overcome this problem by using siRNA to reduce N-CoR levels in human embryonic kidney cells sufficiently to impair repression by TR, in a manner that could be rescued by wild type mouse N-CoR. We then tested the ability of N-CoR RD1, DAD, and HID mutants to rescue repression by TR. Using this strategy, we have ascertained that the DAD plays a unique role in the repressive function of N-CoR.

## Results

**Deletion of DAD but not RD1 abolishes N-CoR-associated HDAC activity.** A series of Flag epitope-tagged wild type (Fig. 1a) and deletion mutants (Fig. 1b) were created to investigate the functions of RD1, DAD, and HID in the context of the N-CoR molecule rather than as isolated domains. The mutants and wild type N-CoR were expressed at similar levels in human embryonic kidney 293T cells, with little effect upon the expression of core components of N-CoR complexes HDAC3, TBL1/TBLR1, and GPS 2 (Fig 1c). As expected, robust HDAC activity co-immunoprecipitated with wild type N-CoR (Fig. 1d). Deletion of the DAD, alone or together with the HID, nearly abolished N-CoR-associated HDAC activity whereas deletion of RD1 ( $\Delta$ 225 as well as  $\Delta$ 312) had little effect (Fig. 1d). As expected, wild type N-CoR co-immunoprecipitated with HDAC3, TBL1, TBLR1, and GPS2 (Fig. 1e). In contrast, neither HDACs 1 and 2, nor Sin3, was co-immunoprecipitated with N-CoR (data not shown). Deletion of the N-terminal 225 amino acids abrogated the interaction with GPS2, and TBL1/TBLR1

interaction was abolished upon further deletion to amino acid 312 (Fig. 1e). DAD deletion abolished HDAC3 interaction, which was also expected. Surprisingly, deletion of the DAD also reduced (but did not abolish) the interactions of TBL1/TBLR1 and GPS2 (Fig. 1e).

The role of the DAD was further explored using a series of point mutants. The DAD of N-CoR/SMRT/SMRTER is highly conserved across different species (Fig. 2a). Previous studies identified residues that perturbed the binding and activation of HDAC3 by the isolated SMRT DAD (41). Based on those studies, we mutated four DAD residues (F448, F459, V471 and Y478) to alanine in the context of full-length N-CoR. All of the mutants expressed in 293T cells at levels similar to wild type without altering the expression of other N-CoR complex components (Fig. 2b). Consistent with predictions from the isolated SMRT DAD, F459A and Y478A almost completely abolished N-CoR associated HDAC activity, F448A led to a modest reduction, and V471A had little effect (Fig. 2c). This correlated with the co-immunoprecipitation of HDAC3 (Fig. 2d). Consistent with earlier results with complete DAD deletion, point mutants that abolished HDAC3 interaction also reduced the association of TBL1, TBLR1 and GPS2 with N-CoR (Fig. 2d), suggesting that the integrity of the DAD and/or the binding of HDAC3 stabilizes the binding of the other core corepressor complex components. Moreover, these results demonstrate that the DAD is required for HDAC3 interaction, and HDAC3 is primarily responsible for the HDAC activity of N-CoR complexes.

**DAD mutations impair the transcriptional repression function of N-CoR.** We next assessed the effect of N-CoR mutations on the transcriptional repression function of N-CoR when fused to the Gal4 DBD (Figure 3). Wild type N-CoR is a very potent repressor in this system (Fig. 3a), and all of the mutants were expressed at similar levels (Fig. 3b). Although RD1 has been shown to have autonomous repressive activity, deletion of RD1 ( $\Delta 225$  and  $\Delta 312$ ) had little effect on the overall repression function of N-CoR. Deletion of the DAD, or point mutations that abrogate HDAC3 interaction (F459A and Y478A), markedly diminished the repressive function of N-CoR;

DAD mutations that did not greatly interfere with HDAC3 interaction (F448A and V471A) had little effect on repression (Fig. 3a). Interestingly, deletion of the HID decreased repression function nearly 40%, and the combined loss of DAD and HID dramatically reduced repression. These data suggest that RD1 is dispensable or redundant for repression by N-CoR, and that the DAD and HID play a major role in repression by N-CoR. In the absence of the DAD and HID, HDAC-independent mechanisms potentially including RD1 or other domains contribute to a relatively modest degree of repression.

**siRNA knockdown of human N-CoR and rescue by mouse N-CoR.** We next aimed to determine the effect of the RD1, DAD, and HID mutations on the function of N-CoR as a TR corepressor. Such studies were previously not possible because N-CoR concentrations are not limiting in most mammalian cells. However, we recently reported that siRNA to human N-CoR specifically knocked-down its target protein and effectively relieved repression by TR in 293T cells (20); there are two mismatches between the human N-CoR siRNA target sequence and the corresponding mouse N-CoR sequence (Fig. 4a), suggesting that the mouse species might not be affected. Indeed, while the siRNA to human N-CoR dramatically reduced the expression of the endogenous proteins, it did not discernibly affect the level of ectopically expressed mouse N-CoR at several concentrations of transfected cDNA (Fig. 4b). Consistent with previous results, the reduction in endogenous N-CoR markedly impaired the repression function of TR (Fig. 4c). Importantly, this was rescued by ectopically expressed mouse N-CoR, in a dose-dependent manner (Fig. 4c).

**A functional DAD is required for N-CoR to act as TR corepressor.** The ability to rescue siRNA-mediated loss of human N-CoR function with mouse N-CoR allowed us to assess the importance of N-CoR repression domains in the context of the full length molecule in its cellular role as TR corepressor. N-CoR RD1 mutants that were shown earlier to abrogate binding of TBL1/TBLR1 and GPS2 were, nevertheless, nearly full functional in replacing endogenous N-

CoR as TR corepressor (Fig. 5a). By contrast, deletion of the DAD essentially destroyed the TR corepressor function of N-CoR (Fig. 5a). Deletion of the HID alone had a more modest effect. Point mutations in the DAD that abolished HDAC3 interaction and N-CoR associated HDAC activity (F459A, Y478A) were also crippled as TR corepressors, whereas the V471A mutant that maintained HDAC3 binding was an effective corepressor (Fig. 5b). The F448A mutant that modestly impaired HDAC3 binding was also an effective corepressor, although its activity may have been subtly reduced. These results demonstrate that the DAD is required for N-CoR to function as a corepressor for TR in human 293T cells.

## **Discussion**

Although several N-CoR-derived polypeptides have ability to repress transcription in the context of heterologous fusion proteins(17), the abundance of endogenous N-CoR has hindered measurements of the relative importance of these RDs, their ability to function in the context of full-length N-CoR, and their role NR corepression. Here, we have taken advantage of the ability to selectively knockdown human N-CoR to study the molecular basis of TR repression. Gal-TR was chosen for study because N-CoR knockdown has a major effect on its repressive ability. We found that most of the cellular HDAC activity associated with N-CoR is due to recruitment of HDAC3, which is completely dependent upon the DAD. The N-CoR DAD, in turn, is essential for TR to function as a powerful repressor of gene transcription.

Early studies suggested that HDAC1 was an important component of NR corepressor complexes (31-33). However, more recent studies have not been consistent with this model: HDAC1 has not been detected in endogenous N-CoR complexes (34, 35), is not recruited to repressed genes along with N-CoR by unliganded TR (20, 46), and is not required for repression by TR (20). The present studies add to the weight of evidence that HDAC3, rather than HDAC1, is the critical mediator of repression by N-CoR.

The DAD has been previously shown to bind to HDAC3 *in vitro* (41). Here we have demonstrated that the DAD is also required for N-CoR to interact with endogenous HDAC3 in cells. N-CoR mutants that fail to bind HDAC3 have little or no detectable HDAC activity. Furthermore, point mutations in the DAD cripple N-CoR's ability to function as TR corepressor in proportion to their inability to bind HDAC3. Thus, unlike earlier studies that studied N-CoR RDs in isolation and independent of NRs, the present studies reveal the singular importance of the DAD in mediating the repression activity of TR. TR was a useful model for this siRNA/rescue approach because of its preference for N-CoR over SMRT. Similar studies of other NRs, as well as other classes of transcription factors that utilize N-CoR as corepressor, are feasible but may require simultaneous knockdown of SMRT.

The critical importance of the DAD is consistent with its strong conservation across species and between N-CoR and SMRT. In comparing the importance of the DAD region with the originally described RDs (17), we focused particularly on RD1 because it has been previously shown to bind to several repressive molecules *in vitro*. One of these is the corepressor Sin3, which is at the core of complexes containing HDACs 1 and 2. Our studies clearly show that this interaction is not critical for N-CoR-associated HDAC activity, nor for the ability of N-CoR to function as TR corepressor.

RD1 also is the main site for interaction with TBL1/TBLR1 and GPS2, which are stoichiometric components of endogenous N-CoR complexes. In the present studies, N-CoR mutants lacking RD1 failed to interact with these proteins yet retained nearly wild type TR corepressor function. This was surprising because TBL1 and TBLR1 have been shown to be required for repression by TR (19). The reason for this discrepancy is unclear, but could relate to different cell types and promoters studied. Another unexpected finding was that DAD mutants that fail to interact with HDAC3 display reduced binding to TBL1/TBLR1 and GPS2. Biochemical studies of the

recombinant proteins have shown that HDAC3 and TBL1 interact independently with SMRT and not with each other. The explanation for this finding is not clear. It suggests that the DAD controls the structural integrity of other polypeptide domains within the N-CoR molecule. It may also be related to the regulated assembly of the HDAC3-N-CoR complex, which involves dissociation of the chaperoning TCP-1 ring complex from HDAC3 (47). Whatever the mechanism, the reduced TBL1/TBLR1 and GPS2 interactions may contribute to the loss of corepressor function of the N-CoR DAD mutants. By the same token, the residual corepressor activity of the DAD mutants that do not associate with HDAC activity points to minor but potentially important functions of the other N-CoR RDs.

Our findings have potentially important therapeutic implications, particularly in myeloid leukemias whose pathogenesis involves aberrant corepressor recruitment. Drugs that inhibit the activity of Class I and Class II HDACs have been successfully used to treat these diseases, but lead to serious side effects (48). The observation loss of HDAC3 interaction abolishes N-CoR associated HDAC activity suggests that a specific inhibitor of HDAC3 enzyme activity might selectively modulate NR corepressor function. The catalytic domains of the Class I and II HDACs are quite similar, and it may not be possible to develop a completely HDAC3-selective inhibitor. The observation that point mutations in the DAD dramatically reduce N-CoR associated HDAC activity suggest that selective therapeutic intervention might also be achieved by pharmacological interference with the DAD-HDAC3 interaction.

## **Methods**

**Plasmids.** pCMX-Gal-DBD, pCMX-Gal-TR $\beta$ 1, pCMX-FLAG-mouse N-CoR constructs have been described previously (20, 25). Deletion constructs were created by standard PCR and cloning procedures. Point mutation constructs were created with Quickchange site-directed mutagenesis kit (Stratagene). The identity of mutations was verified by DNA sequencing.

**Mammalian cell culture and transfection.** 293T cells were maintained in DMED (high glucose) supplemented with 10% fetal bovine serum and L-glutamine (all from GIBCO BRL). Cells were grown at 37 °C in 5% CO<sub>2</sub>. 293T cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For immunoprecipitation, 10 µg of pCMX empty vector or series of pCMX-FLAG-N-CoR constructs were transfected into 293T cells. 48 h after transfection, cells were harvested. For luciferase assay, cells were plated in 24 well plats. 0.1 µg of GAL x 5-SV40-luciferase reporter, indicated amount of Gal4 DBD or Gal4-fusion construct expression vector and 0.01 µg of  $\beta$ -galactosidase expression vector were added to each well. 48 h after transfection, cells were harvested. Luciferase assay kit (Promega) was used to determine relative levels of the luciferase gene product. Light units were normalized to a cotransfected  $\beta$ -galactosidase expression plasmid. Fold repression is relative to the Gal4 DBD, and results of duplicate samples are plotted.

**Immunoprecipitation.** Cells were washed in PBS and lysed with lysis buffer A (150mM NaCl, 40mM Tris-HCL at pH 7.6, 10% glycerol, 0.3% NP-40) and protease inhibitors on ice for 30 min. Lysates were clarified by centrifugation at 12,000g for 10 min at 4°C. Immunoprecipitations were carried out with anti-FLAG agarose beads (Sigma) at 4°C overnight. Pellets were washed three times in lysis buffer A and three times in lysis buffer A containing 300 mM NaCl, 0.1% NP-40 and subjected to immunoblot or HDAC assay.

**HDAC assay.** <sup>3</sup>H-labeled acetylated HeLa histones were prepared essentially as described previously (41). Deacetylase activity of immunoprecipitations was assayed by incubating the pelleted beads with 10,000 cpm of <sup>3</sup>H-labeled acetylated HeLa histones in a total volume of 200µl of HD buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol) at 37°C for 2hr. To stop each reaction, 50 µl of Stop solution (1M HCl, 0.16M HAc) was added and <sup>3</sup>H-labeled acetic acid was extracted with 600 µl of ethyl acetate and measured.

**Immunoblot analysis.** Proteins were separated by SDS-PAGE and transferred to PVDF membranes with HMW transfer buffer (50 mM Tris, 380 mM glycine, 0.1% SDS, and 20% methanol). Blots were probed with the following primary antibodies in TBS containing 5% non-fat dry milk, followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Boehringer Mannheim) and ECL reagent (Amersham): anti-FLAG antibody (Sigma), anti-N-CoR antibody (20), anti-TBL1 antibody (34), anti-TBLR1 antibody (kind gift of Dr. Jiemin Wong, Baylor College of Medicine) and anti-HDAC3 antibodies (Novus). Rabbit polyclonal antibody to GPS2 was raised against a GPS2-derived peptide.

**RNA interference.** The human N-CoR siRNA construct has been described previously (20). Control or N-CoR siRNA constructs were transfected into cells. 24 h after transfection, cells were divided into 24 wells plates with DMEM containing charcoal-dextran stripped fetal bovine serum. After additional incubation for 24 h, second transfection was performed with 0.1  $\mu$ g of GAL UAS x 5 SV40 Luciferase reporter, 0.01  $\mu$ g of  $\beta$ -galactosidase expression vector, 0.1  $\mu$ g of Gal4 DBD or Gal4 fusion constructs expression vector and indicated amount of pCMX or series of mouse N-CoR constructs. Cells were incubated for an additional 48 h and harvested for luciferase assay and immunoblot analysis.

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## Figure Legends

**Figure 1. Deletion of DAD but not RD1 abolishes N-CoR associated HDAC activity.** **a, b.** Schematic representation of FLAG-tagged full-length mouse N-CoR and deletion mutants.  $\Delta$ DAD lacks N-CoR amino acids 312 to 565.  $\Delta$ HID lacks N-CoR amino acids 514 to 685.  $\Delta$ DAD-HID lacks N-CoR amino acids 312 to 685.  $\Delta$ 225 lacks N-CoR amino acids 1 to 225.  $\Delta$ 312 lacks N-CoR amino acids 1 to 312. **c.** Western analysis of N-CoR complex components in 293T cells transfected with mouse N-CoR. **d.** N-CoR associated HDAC activity. **e.** Immunoprecipitated Flag-mouse N-CoR complexes.

**Figure 2. DAD point mutation abolishes N-CoR associated HDAC activity.** **a.** Alignment of DAD from mouse N-CoR (mN-CoR), human N-CoR (hN-CoR), mouse SMRT (mSMRT), human SMRT (hSMRT) and *Drosophila* SMRTER. Identical residues are shown on black. Similar residues are shaded gray. **b.** Western analysis of N-CoR complex components in 293T cells transfected with mouse N-CoR. **c.** N-CoR associated HDAC activity. **d.** Immunoprecipitated Flag-N-CoR complexes.

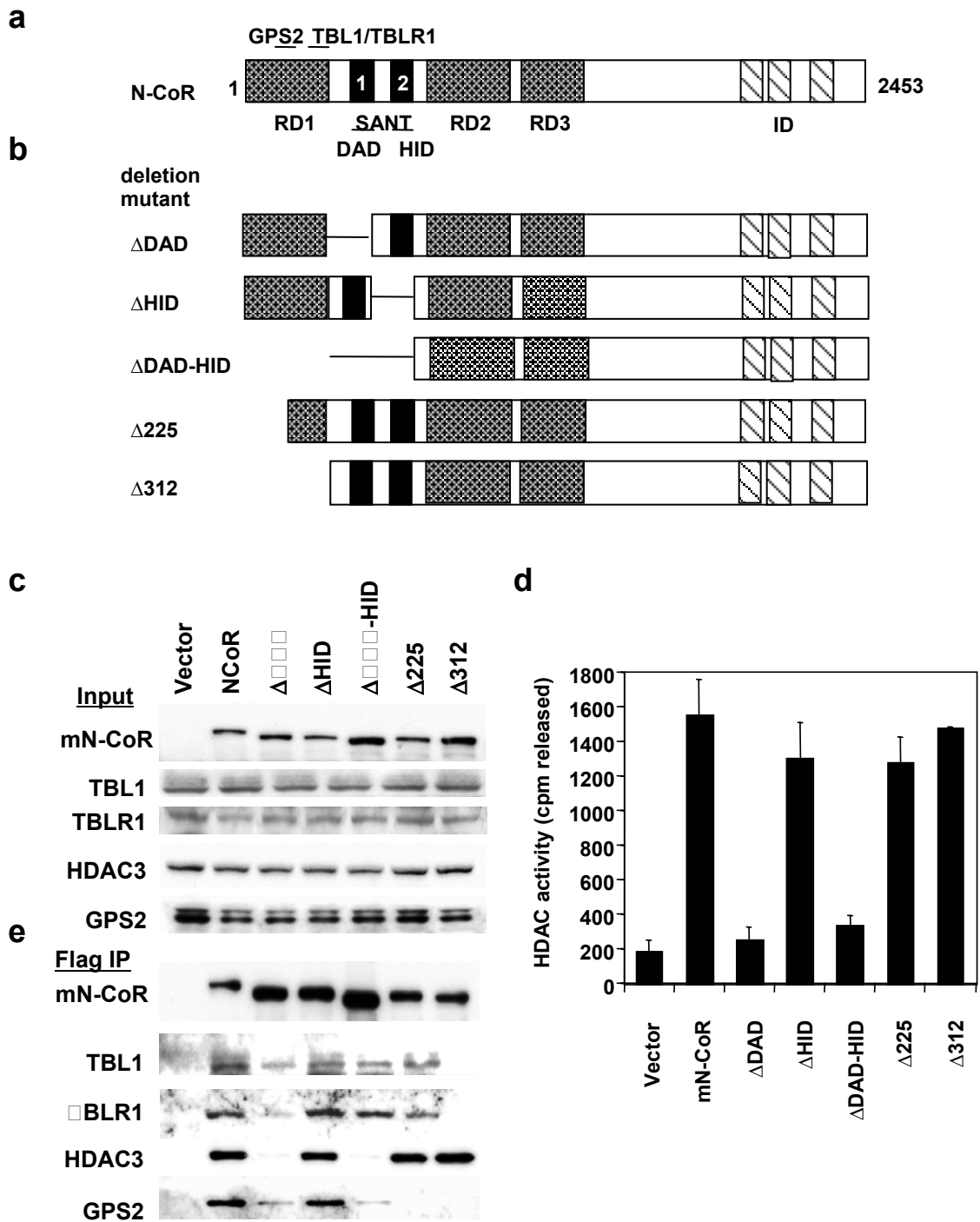
**Figure 3. DAD mutations decrease transcriptional repression function of N-CoR.** **a.** Various N-CoR mutants were fused to Gal 4 DBD and assessed for repression activity in 293T cells. **b.** Immunoblot for Gal fusion N-CoR mutants and HDAC2 (control).

**Figure 4. Knockdown of human N-CoR and functional rescue with mouse N-CoR.** **a.** Sequences of human N-CoR siRNA target and corresponding sequence of mouse N-CoR. Two mismatches are undetlined. **b.** siRNA to human N-CoR specifically knocks down human N-CoR but not ectopically expressed mouse N-CoR. Control or human N-CoR siRNA was transfected along with empty vector or Flag-N-CoR expression vector in different amounts (0.1, 0.2 and 0.4  $\mu$ g). **c.** Rescue of repression function by mouse N-CoR. Gal DBD (0.1  $\mu$ g) or Gal TR (0.1  $\mu$ g)

were transfected along with increasing amounts of CMX or mouse N-CoR (0.1, 0.2 and 0.4  $\mu\text{g}$ ). Luciferase assays were performed 48 hours after transfection. Fold repression is relative to Gal DBD.

**Figure 5. The DAD but not RD1 is required for N-CoR to serve as TR corepressor. a.** After knocking-down hN-CoR, (GAL4 UASx5)-SV40-Luciferase (0.1  $\mu\text{g}$ ),  $\beta$ -galactosidase control vector (0.01 $\mu\text{g}$ ) and either Gal DBD (0.1 $\mu\text{g}$ ) or Gal TR (0.1  $\mu\text{g}$ ) were transfected along with empty vector or mouse N-CoR deletion constructs in increasing amounts (0.1, 0.2 and 0.4  $\mu\text{g}$ ). Luciferase assays were performed 48 hours after transfection. Fold repression is relative to Gal DBD. **b.** Effect of DAD point mutations.

**Figure 1**



**Figure 2**

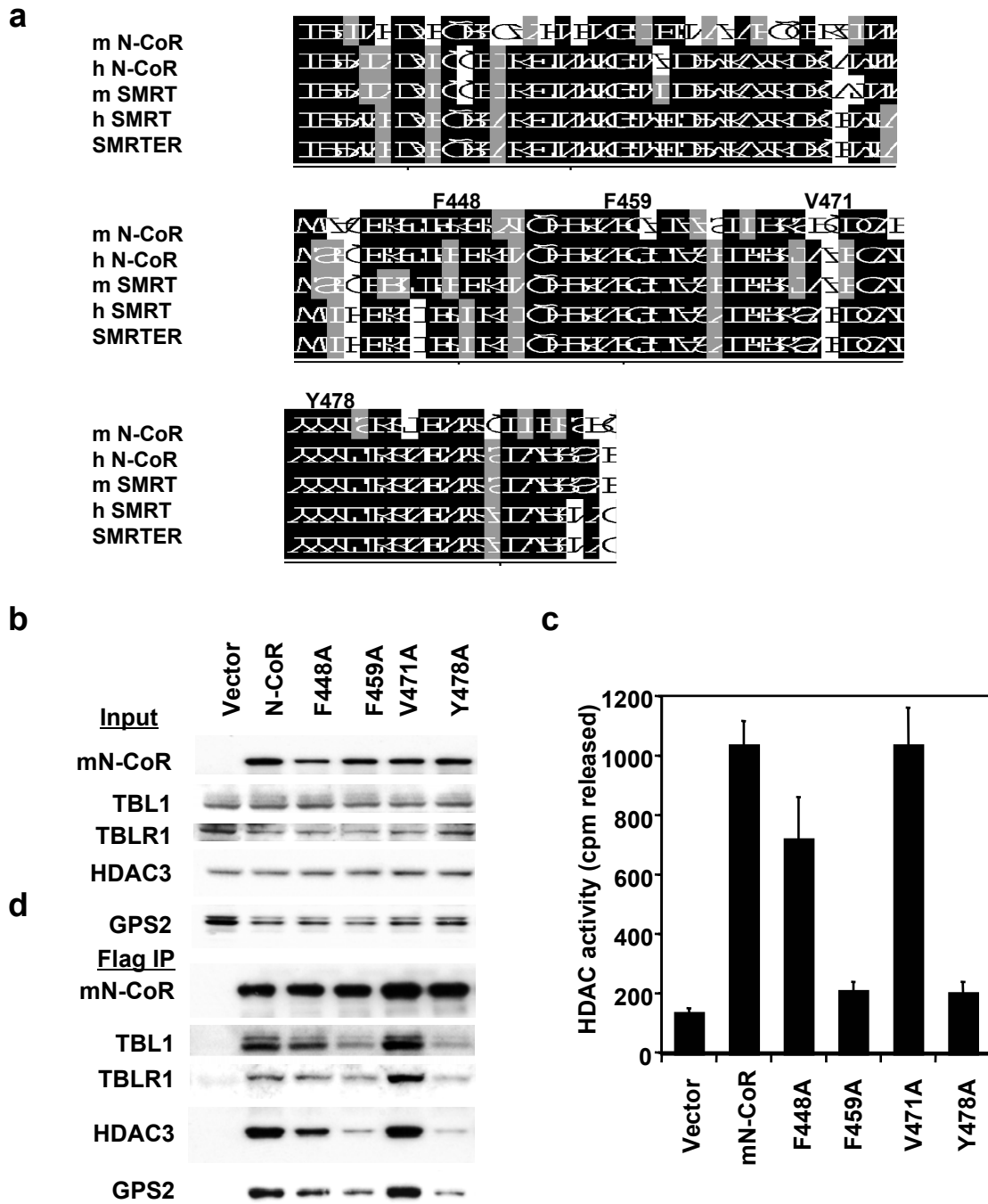
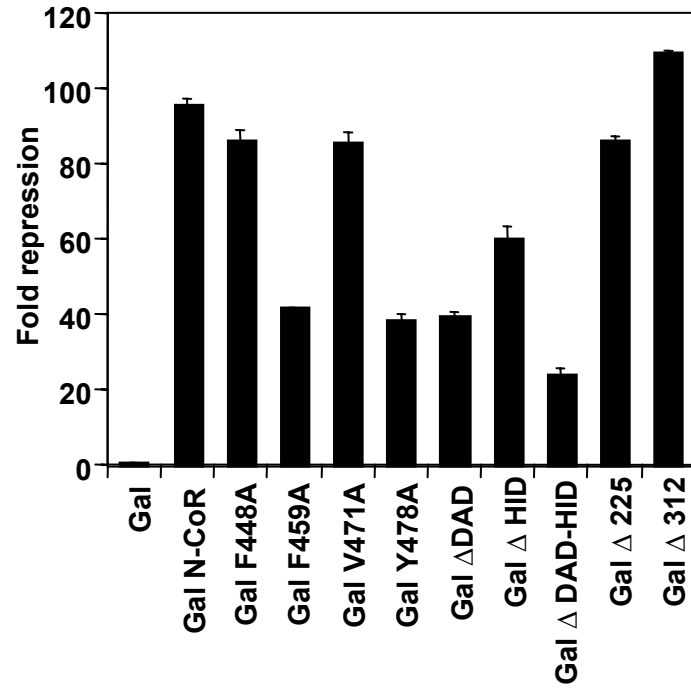
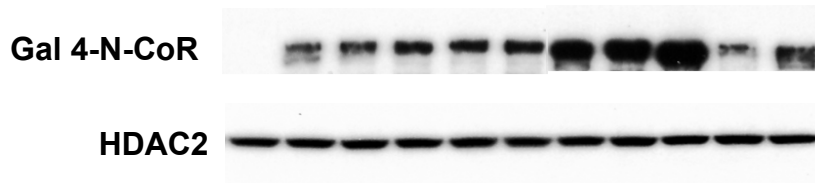


Figure 3

a



b



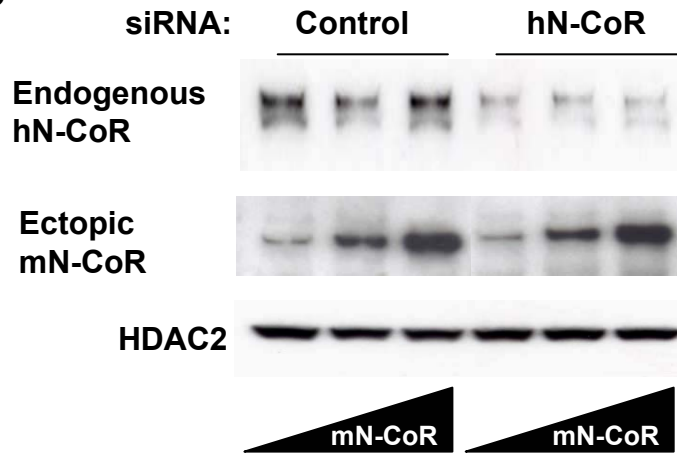
**Figure 4**

**a**

hN-CoR siRNA : 430-AAGAAGGATCCAGCATTTCGGA

mN-CoR: 430-AAGAAGGATCCGGCATTTGGA

**b**



**c**

